蝶と蛾 *Lepidoptera Science* **68**(3/4): 121–128, December 2017

Unique phylogenetic position of the Japanese *Papilio machaon* population in the subgenus *Papilio* (Papilionidae: *Papilio*) inferred from mitochondrial DNA sequences

Misa Miyakawa, Sayoko Ito-Harashima and Takashi Yagi*

Department of Biology, Graduate School of Science, Osaka Prefecture University, 1-2 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8570 Japan

Abstract *Papilio machaon* is distributed in the subarctic and temperate zones of the northern hemisphere, including the Eurasian and North American Continents. It is also distributed in the Japanese Islands and Sakhalin, and is classified as the subspecies *hippocrates*. In order to elucidate the phylogenetic relationship between the Japanese *P. machaon* population and Continental populations, a molecular phylogenetic analysis was performed with mitochondrial *ND5* DNA sequences using *P. machaon* specimens collected from various areas in the Japanese Islands and foreign countries and other species included in the subgenus *Papilio*. We found that the Japanese *P. machaon* population (the subgenus *hippocrates*) was genetically distinct from the Eurasian and North American populations. The Japanese population diverged earlier than other Continental *P. machaon* populations in the subgenus *Papilio*, which indicates that the Japanese population would be isolated in the Islands since their geographical establishment. These results imply that the Japanese population of other butterfly species may also be distinct from the Continental populations at the molecular level even though morphological similarities exist between the populations.

Key words biogeography, mitochondria DNA, molecular phylogeny, *Papilio machaon*, speciation.

Introduction

The genus *Papilio* Linnaeus, 1758 consists of various morphologically distinct groups, some of which have been further classified into subgenera such as *Papilio*, *Euchenor*, *Menelaides*, and *Achillides* (Igarashi, 1979). The subgenus *Papilio* includes approximately 10 species that are distributed in the northern hemisphere (Fujioka *et al.*, 1997). *Papilio machaon*, *P. hospiton*, *P. saharae*, and *P. sikkimensis* are distributed in the Eurasian Continent and its affiliated areas (Fujioka *et al.*, 1997), while various species such as *P. machaon*, *P. polyxenes*, *P. zelicaon*, *P. indra*, *P. brevicauda*, *P. hudsonianus*, *P. oregonius*, and *P. bairdii* are distributed in the North American Continent (Scott, 1986; Fujioka *et al.*, 1997). The classification of these species is complex because some hybridize in their common habitats (Dupuis and Sperling, 2015; 2016), and some of them are regarded as subspecies by some authors (Tyler, 1994 ; Fujioka *et al.*, 1997). *Papilio machaon* is widely distributed in the subarctic and temperate zones from Europe to northern America through Russia, Mongolia, China, Alaska, and Canada, and many subspecies have been recorded in various regions. In east Asian islands including Japan, *P. machaon* is distributed in Sakhalin, Hokkaido, Honshu, Shikoku, Kyushu, and their affiliated small islands (Fujioka *et al.*, 1997), and this population has been classified as the subspecies *hippocrates* C. et R. Felder, 1864.

*Corresponding author, yagi-t@riast.osakafu-u.ac.jp, +81-72-254-9862

Papilio machaon is one of the common papilionid butterflies in the Japanese Islands, including Sakhalin, and is distributed in wide areas, except for the southern Ryukyu islands. The difference in latitude between the northernmost and southernmost habitats, Sakhalin and Yakushima Islands, respectively, is approximately 24 degrees. According to the records of the Japan Meteorological Agency (2017), the difference in the average annual temperature between these islands is approximately 17℃. Larval host plants of *P. machaon* are various local species of *Umbelliferae*. In the middle area of Honshu, their larvae were found at the seashore as well as in mountains with an altitude of 2,000 m. These findings indicate that *P. machaon* is adapted to highly variable climate conditions in the Japanese Islands.

Previous studies elucidated the molecular phylogenetic relationships among species of the subgenus *Papilio* (Spering and Harrison, 1994; Aubert *et al.*, 1999; Caterino and Sperling, 1999 ; Reed and Sperling, 1999 ; Zakharov *et al.*, 2004); however, the Japanese population was not examined in detail. Many types of endemic insects including butterflies such as *Luehdorfia japonica*, *Lethe sicelis*, and *Neope goschkevitschii* inhabit the Japanese Islands (Kawazoe and Wakabayashi, 1976; Shirôzu, 2006). In some butterfly species inhabiting the Japanese Islands and Eurasian Continent, e.g., *Parnassius citrinarius*, *Artogeia napi*, and *Erebia niphonica*, the Japanese population is largely divergent from the Continental populations at the molecular level (Yagi *et al.*, 2001 ; Fujii *et al.*, 2001 ;

Nakatani *et al.*, 2007 ; Shinkawa and Nonaka, 2010). These differences in molecular phylogeny may reflect the geographical isolation of the Japanese Islands since their formation (Tojo and Ito, 2015). The repeated connection and separation of the Islands at the eastern edge of the Eurasian Continent during the late Pliocene and Pleistocene resulted in the unique characteristics of butterfly fauna in the Japanese Islands (Tojo and Ito, 2015).

Papilio machaon is a widely distributed species in the northern hemisphere, whereas the Japanese Islands population has unique characteristics in terms of climate adaptation. Genetic differences in the Japanese population from those in other areas are of great interest to entomologists. In the present study, *P. machaon* individuals were collected at various areas in the Japanese Islands including Sakhalin, and the nucleotide sequences of their mitochondrial NADH dehydrogenase subunit 5 (*ND5*) genes were elucidated. Molecular phylogenetic trees of *P. machaon* individuals from the Japanese Islands and foreign areas were constructed with other species of the subgenus *Papilio* in order to clarify phylogenetic relationships with the Japanese population in the subgenus *Papilio*.

Materials and methods

Sample collection

Specimens of *P. machaon hippocrates* were collected in various areas of Hokkaido, Honshu, Shikoku, Kyushu, and their affiliated small islands in Japan between 2006 and 2009. Foreign specimens were collected between 1996 and 2003. Adult bodies without wings and larvae were preserved in 99.5% ethanol at -20℃. Foreign samples that we had previously described (Yagi and Fujioka, 2005) were used for molecular phylogenetic analyses in the present study. Specimens from Japan and foreign countries were kindly provided by many entomologists. Species, sampling locations, and database accession numbers are shown in Table 1.

DNA extraction, ND5 gene amplification, and sequencing

Adult legs or larval muscles were shredded by scissors in a test tube, and mixed with 500 μl lysis solution containing 0.1% SDS and 0.5 mg/ml proteinase K. The test tube was then kept on a rotator for 30-120 min at 55℃. The digested samples were mixed with phenol once, phenol/chloroform (1 : 1) twice, and chloroform/isoamylalcohol (24 : 1) once. DNA in the water fraction was precipitated with ethanol, washed twice with 75% ethanol, and dried. Dried DNA was resuspended in 50 μl Tris-EDTA buffer (pH 7.5) and stored at -20℃.

A part of the *ND5* gene of mitochondrial DNA was amplified with a polymerase chain reaction (PCR) using the following primers: V 1 (5'-CCTGTTTCTGCTTTAGTTCA-3'), K3 (5' -TAKCTTCAATATTAYRCTCT-3') (Yagi *et al.*, 1999), and KIAGEHA (5'-TAGGACAAAGTTTATTAAAG- 3'). The PCR reaction mixture contained 25 μl buffer, 10 μl dNTPs, 1.5 μl V1 primer (10 μM), 1.5 μl K3 primer (10 μM), 10 μl sterilized water, 1 μl butterfly DNA (~ 4 ng), and 1 μl DNA polymerase (KOD FX, TOYOBO, Osaka, Japan). PCR reactions were performed using the following amplification profile: 94℃ for 2 min, followed by 30-40 cycles at 98℃ for 10 sec, at 45-50℃ for 30 sec, at 68℃ for 1 min, and final extension at 68℃ for 5 min. The PCR product was inspected by 1% agarose gel electrophoresis, and purified using the Sephacryl S-300 High Resolution column (GE Healthcare, Little Chalfont, UK).

A DNA sequencing reaction of the PCR product was performed with the same primers for PCR and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Waltham, MA). DNA sequences were obtained with the ABI 3100 Genetic Analyzer (Applied Biosystems, Waltham, MA).

DNA primers were synthesized by Sigma-Aldrich Japan, Tokyo, and other chemicals were purchased from Nacalai Tesque, Kyoto; TAKARA BIO, Kusatsu; and Sigma-Aldrich Japan, Tokyo.

Phylogenetic analysis

ND5 gene nucleotide sequences (717 bp) were aligned using Clustal W and fragment comparisons were conducted using BioEdit version 7.0.9.1 (Hall, 1999). A phylogenetic analysis of *P. machaon* samples obtained at various locations in the Japanese Islands and other countries was conducted using the Maximum likelihood (ML) method and Neighbor-joining (NJ) method with MEGA5 software (Tamura *et al*., 2011). In the analysis, evolutionary history was inferred using the Tamura-Nei model (Tamura and Nei, 1993) with a uniform base substitution rate and bootstrap of 10,000 replicates. The codon positions included were 1st+ 2nd+ 3rd+Non-coding. The base substitution was confirmed to be unsaturated by the function included in MEGA5. The ML tree was drawn with the highest log likelihood values and a scale of the branch length proportional to the number of substitutions per site. The NJ tree was drawn with the values of bootstraps and branch lengths higher than 0.003.

Results and Discussion

The ML and NJ molecular phylogenetic trees of the subgenus *Papilio* were shown in Figures 1 and 2 , respectively. When *P. xuthus* and *P. benguetana* were settled as outgroup species, *P. indra* diverged first; the pair of *P. polyxenes* and *P. zelicaon* diverged second; the pair of *P. brevicauda* and *P. hudsonianus* diverged third; and the *P. machaon* group finally diverged. Among *P. machaon*, it is important to note that the Japanese population (clade V: subspecies *hippocrates*) diverged first. The divergence order of other *P. machaon* populations currently remains unclear due to low bootstrap values; however, *P.*

Table 1. *Papilio* samples used in the phylogenetic analysis.

Species	Haplo-type	Accession number	Location collected	Clade*
P. machaon	K1	LC061680	Japan: Hokkaido: Esan, Honshu: Omonogawa, Gojome, Oga, Kisakata, Sabusawashima, Katsurashima, Shichigahama, Yuza, Sagae, Mito, Tainai, Ito, Shimoda, Tatsunokuchi, Hakusan, Mikata, Joyo, Awajishima, Maniwa, Yamaguchi, Shikoku: Miyoshi, Marugame, Manno, Kyushu: Iizuka, Kanzaki, Aso, Tsushima; Korea: Jeju-do	V
P. machaon	K2	LC061681	Japan: Honshu: Oma, Oga, Kisakata, Sagae, Mito, Joyo, Maniwa, Shikoku: Miyoshi, Manno, Kyushu: Iizuka, Aso	V
P. machaon	F2	LC061665	Japan: Hokkaido: Wakkanai, Kushiro, Furano, Esan, Rishiri, Rebun; Russia: Sakhalin	V
P. machaon	W1	LC061725	Japan: Hokkaido: Wakkanai, Furano, Esan, Honshu: Oma; Russia: Sakhalin, Magadan	V
P. machaon	SY3	LC061717	Russia: Sakhalin	V
P. machaon	K20	LC061684	Japan: Honshu: Shimoda, Hakusan, Joyo	V
P. machaon	SH1	LC061706	Japan: Honshu: Kisakata, Shimoda, Yamaguchi	V
P. machaon	T9	LC061720	Japan: Honshu: Oma, Sabusawashima, Sagae, Mito, Tainai, Hakusan	V
P. machaon	AE	LC061736	Russia: Magadan, Chita, Kamchatka, Tuva, East Sayan; Tajikistan: Pamir Mts; Mongolia: Terelj, Tsenkher; China: Gansu; Macedonia; USA: California, Oregon: Spensbridge; Canada: Yukon	I
P. machaon	AF	LC061737	Russia: Kamchatka	I
P. machaon	AG	LC274577	USA: California: San Bernardino	I
P. machaon	AH	LC274578	Russia: East Sayan	I
P. machaon	AI	LC061738	Russia: Chita	I
P. machaon	AJ	LC061739	Russia: Primorsky Krai	I
P. machaon	AK	LC274579	Russia: Altai	I
P. machaon	AL	LC274580	Canada: Yukon	I
P. machaon	AM	LC061740	China: Yunnan: Mt Guangya	I
P. machaon	L	LC061730	Korea: Gyeonggi-do: Yonjongdo	II
P. machaon	W	LC061733	China: Yunnan	II
P. machaon	X	LC274581	Russia: Altai	II
P. machaon	Z	LC274582	Tajikistan: Khodza-Mumin	II
P. machaon	AA	LC274583	Russia: Orenburg	II
P. machaon	AB	LC061734	Russia: Primorsky Krai	II
P. machaon	AC	LC061735	China: Beijing	II
P. saharae	AV	LC274584	Morocco: Tezenakht	III
P. saharae	AU	LC274585	Morocco: Tezenakht	III
P. sikkimensis	AN	LC061732	China: Tibet	IV
P. sikkimensis	AO	LC274586	China: Tibet	IV
P. sikkimensis	AP	LC274587	China: Tibet	IV
P. brevicauda	U	LC274588	Canada: Newfoundland	
P. hudsonianus	V	LC274589	Canada: Hudson Bay	
P. polyxenes	O	LC061731	Canada: Ontario: Komoka, Manitoba: Duck Mountain	
P. zelicaon	R	LC274590	USA: Idaho	
P. indra	E	LC274591	USA: Colorado: Sweet Pass	
P. benguetana	D	LC274592	Philippines: Benguet	
P. xuthus	G	LC274593	Korea: Gyeonggi-do: Yonjongdo; Japan: Honshu: Kyoto	

*indicates the clade of *P. machaon* phylogenetic trees (Fig. 1 and 2).

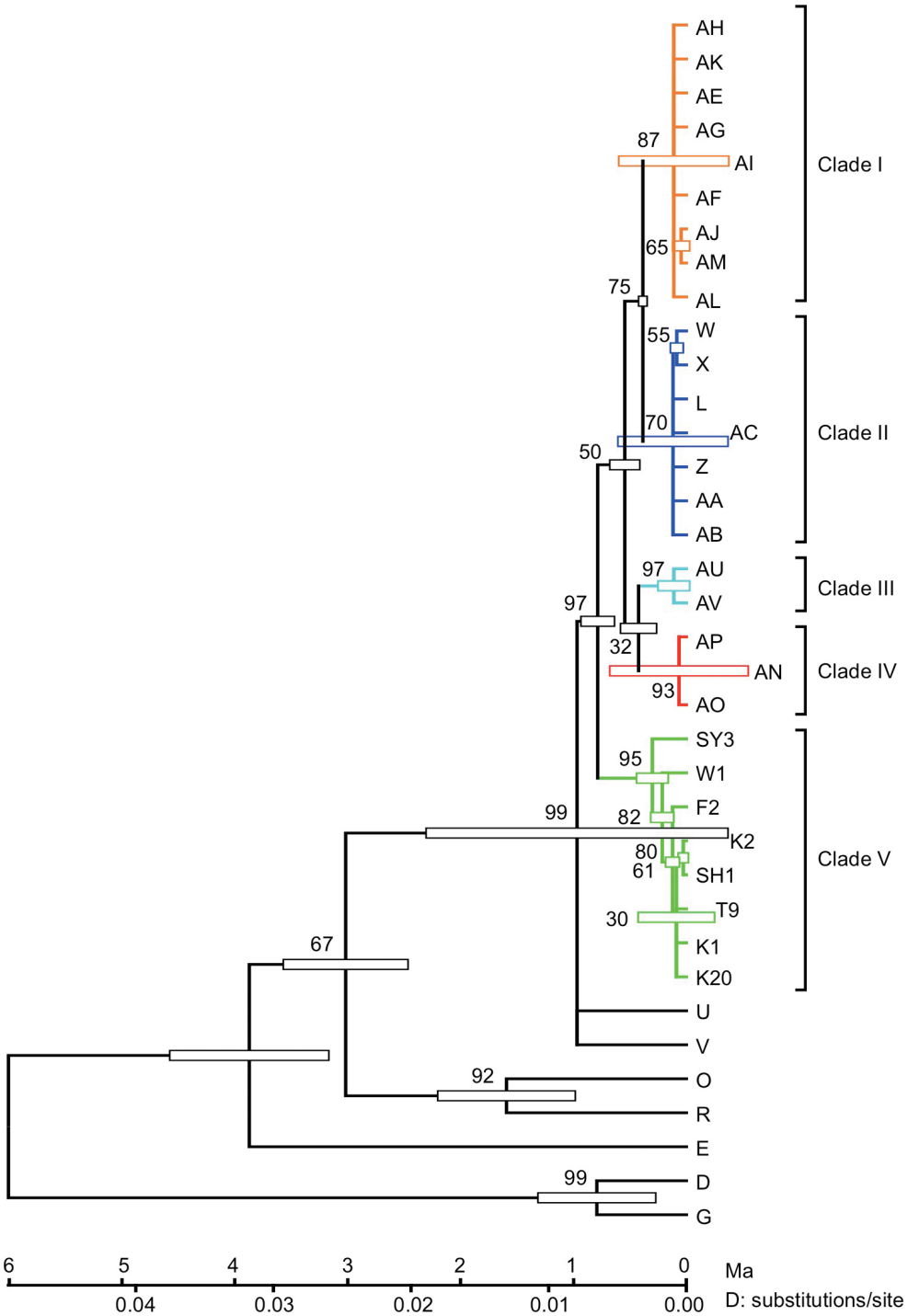

Fig. 1. *ND5* phylogenetic tree of the subgenus *Papilio* including *P. machaon* collected at various locations in the Japanese Islands and other countries, constructed with the Maximum likelihood (ML) method using MEGA5 software. The time scale is based on the tentative evolution rate estimated from *Papilio binor ND5* (0.81% substitutions/site per m.y.) (Osozawa *et al.*, 2013).

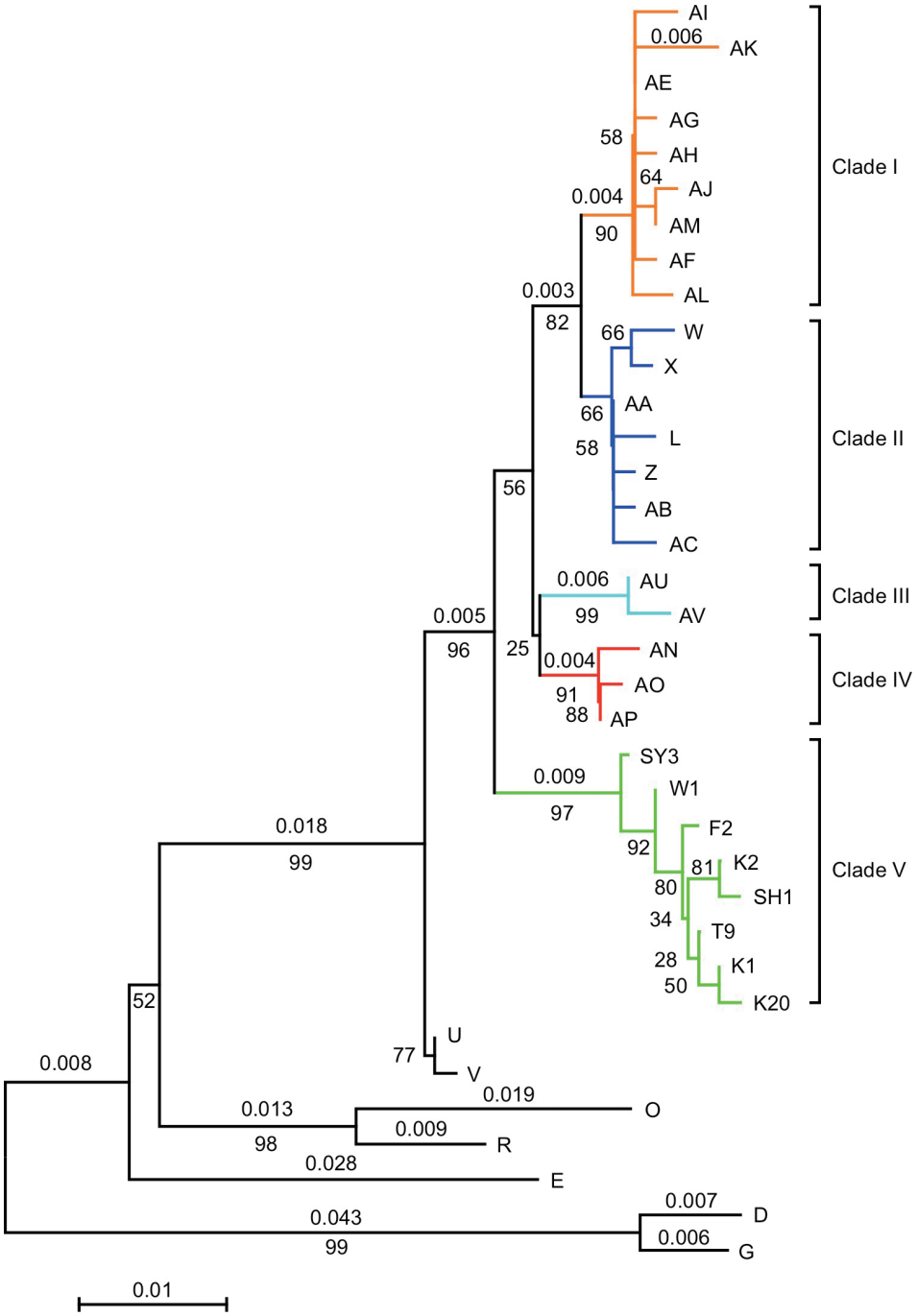

Fig. 2. *ND5* phylogenetic tree of the subgenus *Papilio* including *P. machaon* collected at various locations in the Japanese Islands and other countries, constructed with the Neighbor-joining (NJ) method using MEGA5 software.

machaon was clearly divided into 5 clades. Clade I comprised 25 individuals of the Eurasian and North American Continents with 9 haplotypes. Clade II included 9 individuals of Eurasia with 7 haplotypes. Clade III contained 6 individuals of *P. saharae* from northern Africa. Clade IV comprised 3 individuals of *P. sikkimensis* from Tibet. Clade V comprised 312 individuals of *P. machaon* from the Japanese Islands, Sakhalin, and Jeju-do Island (Korea) with 8 haplotypes. A similar phylogenetic topology was obtained by the ML and NJ methods (Fig. 1 and 2). In the Eurasian Continent, Clade I and II individuals were distributed sympatrically. As reported by Fujioka *et al*. (1997), *P. saharae* and *P. sikkimensis* are different species from *P. machaon*, but are included in *P. machaon* in these phylogenetic trees (Fig. 1 and 2).

The first divergence of *P. indra* among the subgenus *Papilio* in our *ND5* trees is consistent with previous findings in which the ML and MP (maximum parsimony) phylogenetic trees were constructed using mitochondrial *ND1* and *COI/COII* genes (Aubert *et al.*, 1999 ; Caterino and Sperling, 1999 ; Reed and Sperling, 1999), and the pairing of *P. zelicaon* with *P polyxenes* in our ML and NJ trees was also reported in previous studies (Caterino and Sperling, 1999 ; Reed and Sperling, 1999); however, the divergence order of *P. indra*, the *P. zelicaon* and *P. polyxenes* pair, *P. hospiton*, and *P. machaon* was controversial. In recent studies by Sperling and his colleagues (Zakharov *et al.*, 2004 ; Dupuis and Sperling, 2015), they constructed more concrete ML and Bayesian phylogenetic trees using the *COI/COII* genes with *P. xuthus* as an outgroup species, which showed that divergence occurred in the order of *P. indra*, the *P. zelicaon* and *P. polyxenes* pair, *P. hospiton*, and finally the *P. machaon* species group. Our present results support this divergence order. *Papilio hospiton* is not included in the present study, but discussed in more detail below.

All samples of North American *P. machaon* examined were involved in Clade I (Fig. 1 and 2). In the North American Continent, *P. machaon* has been classified into many species/subspecies, *e.g.*, *P. m. aliaska*, *P. bairdii*, *P. brevicauda*, *P. m. dodi*, *P. hudsonianus, P. joanae*, *P. m. kahli*, *P. m. oregonius*, and *P. m. pikei*, among which genetic introgression among some species/subspecies has been reported (Dupuis and Sperling, 2015; 2016). Based on the results of the present study, we were unable to elucidate phylogenetic relationships among North American *P. machaon* because of the small number of analyzed samples.

Regarding *P. m. hippocrates*, Sperling and his colleagues used only a few samples from Japan in their phylogenetic analysis, and showed that it diverged earlier than other *P. machaon* subspecies from France and Washington, USA (Sperling, 1993; Zakharov *et al.*, 2004). Our present (clade V) and early pilot phylogenetic studies (Yagi and Fujioka, 2005) using a large number of *P. m. hippocrates* samples from various areas of the Japanese Islands and Sakhalin supported the findings of Zakharov *et al.* (2004). *P. m. hippocrates* was clearly distinct from other *P. machaon* subspecies at the molecular level, suggesting vicariant speciation due to its isolation in the Japanese Islands for a few million years. It is currently inconclusive whether the Japanese population is an independent species from Eurasian and North American *P. machaon* due to the difficulties associated with massive hybridization experiments; however, Ae (1971; 1988) reported an unbalanced F_1 sex ratio and low F_1 fertility after the subspecies-crossing of Japanese *hippocrates* with German *gothica* and British *britanicus*. Ae (1988) found that the eggs from the F_1 (*hippocrates* x *britanicus*) mutual cross (F_2 eggs) and F_1 male back-cross with female *hippocrates* rarely hatched. It is important to note that Remington (1959) raised the subspecies *hippocrates* to species because of differences in morphological characteristics and chromosome numbers from European *P. machaon* and a marked deficiency in females in the broods of F_1 hybrids with European *P. machaon*. The major chromosome numbers of Japanese and European *P. machaon* were previously reported to be 62 and 60 , respectively (Maeki, 1953 ; 1976). Phylogenetic analyses performed by Sperling and our groups in addition to previous findings clearly indicated that the Japanese *P. machaon* population (*hippocrates*) shows large genetic differences from the other populations.

Papilio hospiton is endemic to the Mediterranean Sardinia and Corsica Islands, which are located near the Italian Peninsula. Although *P. hospiton* was not used in the present analysis, previous studies showed that it diverged earlier than all *P. machaon* subspecies in phylogenetic trees of the subgenus *Papilio* (Sperling, 1993 ; Zakharov *et al.*, 2004 ; Dupuis and Sperling, 2016). *Papilio hospiton* was found to be an independent species from *P. machaon* based on hybridization and the findings of molecular studies; however, the low frequency of genetic introgression has now been observed between the two species in the field (Cianchi *et al.*, 2003). *Papilio hospiton* may have been isolated in the Sardinia and Corsica Islands for a long time without genetic exchange with Continental *P. machaon*. A similar relationship between Japanese (*hippocrates*) and Continental *P. machaon* has been suggested.

The present *Papilio* phylogenetic trees using mitochondrial *ND5* gene may not be consistent entirely with speciation history of this group because genetic introgression occurs among the North American species/subspecies group (Dupuis and Sperling, 2015; 2016). Although *P. hudsonianus* (V) is generally recognized as a subspecies of *P. machaon* (Scott, 1986), it clustered with *P. brevicauda* (U) (Fig. 1 and 2), which may be due to the mitochondrial DNA introgression. Future work with nuclear genes, either gene sequences, microsatellites, or genome-wide SNPs, will help to elucidate the species phylogeny in this group (Dupuis and Sperling 2016).

In conclusion, we found that the Japanese *P. machaon* population classified as the subspecies *hippocrates* is genetically distinct from the Eurasian and North American populations. Japanese *P. m. hippocrates* diverged earlier than other Continental *P. machaon* subspecies within the subgenus *Papilio*, which indicates that the Japanese population was isolated in the Islands since their geographical establishment. Molecular variations in *P. machaon* within the Japanese Islands need to be analyzed further in order to elucidate phylogeography in more detail. The results of the present study imply that the Japanese populations of other species are also distinct from the Continental populations at the molecular level even though their morphological characteristics are similar between populations.

Acknowledgments

The authors sincerely thank Dr Tomoo Fujioka for providing many Japanese and foreign samples and the valuable comments throughout this study. The authors also thank the following scientists for kindly providing samples: Dr Takatoshi Nakatani and Dr Tateo Ito for the Russian and US samples; Dr Jun-ichi Asahi for the Sakhalin samples; Dr Bong-Woo Lee, Dr Cheol-Min Lee, and Dr Neung-Ho Ahn for the Korean samples; Mr E. Y. Huang and Mr Ken Throne for the Eurasian and American samples; and Dr Hideshi Naka, Dr Hisashi Fujii, Dr Katsuhisa Ozaki, Mr Katsumi Ono, Dr Masakado Kawata, Mr Masayasu Tanaka, Mr Tamotsu Nomura, and Mr Yoshinori Iemura for the Japanese local samples. The authors are indebted to Ms Chiaki Shimohara for her technical assistance. This research was supported by a grant from the Institute of Applied Optics (2001-2006), and by a basic research grant from Osaka Prefecture University (2000-2013).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

Ae, S., 1971. A further study of interspecific hybrids in *Papilio*. *Spec*. *Bull*. *Lep*. *Soc*. *Jpn*. **5**: 89-114. (in Japanese with English summary)

Ae, S., 1988. A study of species and speciation based on hybridization mainly in *Papilio*. *Spec*. *Bull*. *Lep*. *Soc*. *Jpn*. **6**: 475- 498 . (in Japanese with English abstract)

Aubert, J., L. Legal, H. Descimon, and F. Michel, 1999. Molecular phylogeny of swallowtail butterflies of the tribe Papilionini (Papilionidae, Lepidoptera). *Mol*. *Phylogenet*. *Evol*. **12**: 156 - 167.

Caterino, M.S., and F.A. Sperling, 1999. *Papilio* phylogeny based on mitochondrial cytochrome oxidase I and II genes. *Mol*. *Phylogenet*. *Evol*. **11**: 122-137.

Cianchi R., A. Ungaro, M. Marini, and L. Bullini, 2003. Differential patterns of hybridization and introgression between the swallowtails *Papilio machaon* and *P. hospiton* from Sardinia and Corsica islands (Lepidoptera, Papilionidae). *Mol*. *Ecol*. **12**: 1461-1471.

Dupuis J.R., and F.A. Sperling, 2015. Repeated reticulate evolution in North American *Papilio machaon* group swallowtail butterflies. *PLoS One* **10**: e0141882.

Dupuis, J.R., and F.A. Sperling, 2016. Hybrid dynamics in a species group of swallowtail butterflies. *J. Evol*. *Biol*. **29**: 1932-1951.

Fujii, S., T. Shinkawa, and T. Yagi, Phylogenetic relationship among *Pieris-Artogeia* butterflies with a mitochondrial gene. *Nature and Insects* **36**(13): 13-15. (in Japanese)

Fujioka, T., H. Tsukiyama, and H. Chiba, 1997. Japanese butterflies and their relatives in the world I. Shuppan Geijutu Co. Ltd., Tokyo (in Japanese with English summary)

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser*. **41**: 95–98

Igarashi, S., 1979. Papilionidae and their early stages. Kodansha Co. Ltd., Tokyo. (in Japanese)

Japan Meteorological Agency, 2017. Record of the past meteorological data in Japan. http://www.data.jma.go.jp/obd/stats/etrn/index.php. (accessed on January 5, 2017)

Kawazoe, A. and M. Wakabayashi, 1976. Coloured Illustrations of the butterflies of Japan. Supervised by Shirôzu T. Hoikusha Publishing Co. Ltd. Osaka. (in Japanese)

Maeki, K., 1953. Chromosome numbers of some butterflies (Lepidoptera-Rhopalocera). *Jpn. J. Genet.* **28**: 6-7.

Maeki, K., 1976. On the chromosome of *Papilio machaon hippocrates* C. et R. Felder. *Trans. Lepid. Soc. Jpn.* **26**: 85-90. (in Japanese with English summary)

Nakatani T., S.I. Usami, and T. Itoh, 2007. Molecular phylogenetic analysis of the *Erebia aethiops* group (Lepidoptera, Nymphalidae). *Trans. Lepid. Soc. Jpn.* **58**: 387-404.

Osozawa, S., Z.-H. Su, Y. Oba, T. Yagi, Y. Watanabe, and J. Wakabayashi, 2013. Vicariant speciation due to 1.55 Ma isolation of the Ryukyu islands, Japan, based on geological and GenBank data. *Entomol*. *Sci*. **16**: 267-277.

Reed, R.D., and F.A. Sperling, 1999. Interaction of process partitions in phylogenetic analysis: an example from the swallowtail butterfly genus *Papilio*. *Mol*. *Biol*. *Evol*. **16**: 286-297.

Remington C.L., 1959. Wide experimental crosses between *Papilio xuthus* and other species. *J. Lepid. Soc.* **13**: 151-164.

Scott, J.A., 1986. The butterflies of North America. Stanford University Press, CA. pp. 160-190.

Shinkawa, T. and I. Nonaka, 2010. Molecular systematics and evolution of *Pieris napi* group (Lepidoptera; Pieridae). *Nature Insects* **45**(13): 20-26. (in Japanese)

Shirôzu T., 2006 . The standard of butterflies in Japan. Gakken, Tokyo. (in Japanese)

Sperling F.A., 1993. Mitochondrial DNA phylogeny of the *Papilio machaon* species group (Lepidoptera: Papilionidae). *Mem*. *Ent*. *Soc*. *Can*. **165**: 233-242.

Spering, F.A., and R.G. Harrison, 1994. Mitochondrial DNA variation within and between species of the *Papilio machon* group of swallowtail butterflies. *Evolution* **48**: 408-422.

Tamura, K., and M. Nei, 1993. Estimation of the number of

nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Bio. Evol.* **10**: 512 – 526.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar, 2011 . MEGA5 : molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**: 2731–2739.

Tojo K. and T. Ito, 2015. Japanese topography and insect fauna formation—evolutionary world due to geographical development. In Oba, Y. *et al*.（eds）Wonderful world of insects clarified from genes. Yushokan Co. Ltd., Tokyo. pp. 105-150.（in Japanese）

Tyler, H.A., K.S. Jr. Brown, K.H. Wilson, 1994. Swallowtail Butterflies of the Americas: A Study in Biological Dynamics, Ecological Diversity, Biosystematics, and Conservation. Scientific Pub. Inc., Jodhpur, India.

Yagi, T., G. Sasaki, and H. Takebe, 1999. Phylogeny of Japanese papilionid butterflies inferred from nucleotide sequences of the mitochondrial *ND5* gene. *J. Mol. Evol.* **48**: 42-48.

Yagi, T., T. Katoh, A. Chichvarkhin, T. Shinkawa, and K. Omoto, 2001. Molecular phylogeny of butterflies *Parnassius glacialis* and *P. stubbendorfii* at various localities in East Asia. *Genes Genet. Syst*. **76**: 229-234.

Yagi, T., and T. Fujioka, 2005. Classification and molecular phylogeny of subgenus *Papilio*. *Nature and Insects* **40**（4）: 39-42.（in Japanese）

Zakharov, E.V., M.S. Caterino, and F.A. Sperling, 2004. Molecular phylogeny, historical biogeography, and divergence time estimates for swallowtail butterflies of the genus *Papilio*（Lepidoptera: Papilionidae）. *Syst. Biol*. **53**: 193-215.

摘　要

キアゲハはユーラシア大陸および北アメリカ大陸の亜寒帯および温帯地方に広く分布する．キアゲハは日本列島およびサハリンにも分布し，それらは亜種 *hippocrates* として区別される．キアゲハの日本列島（サハリンを含む）集団と大陸集団の系統関係を明らかにするために，国内外諸地域のキアゲハおよびその近縁種から DNA を抽出し，ミトコンドリア DNA の *ND5* 遺伝子の一部塩基配列を決定し，分子系統樹を描いた．その結果，日本列島集団はユーラシア大陸集団および北アメリカ集団から遺伝的に明確に区別されることがわかった．キアゲハの分子系統樹において，日本列島集団はユーラシア大陸集団と北アメリカ集団より先に分かれていた．このことは，キアゲハが，大陸から分かれた日本列島において，長期間大陸集団と交流なく隔離されていたことを示唆している．日本列島集団と大陸集団との間で斑紋が似ていても遺伝的に離れている種が，他にも存在するかもしれない．

（Received June 14, 2017. Accepted July 30, 2017）